DEVELOPMENT OF REPLICATION-COMPETENT VESICULAR STOMATITIS VIRUS SPECIFIC FOR BREAST CANCER CARCINOMA CELLS

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The goal of this project is to develop a replication competent, cytolytic virus that has a tropism for breast carcinoma cells based on the specificity of the viral attachment protein. This will be accomplished by 1) mutating the vesicular stomatitis virus (VSV) surface "G" protein and selecting a variant that has lost binding function but retained fusion function and then 2) constructing a new VSV virus in which the wild-type (wt) G gene is replaced by a gene coding for a chimera which contains the transmembrane (TM) domain and fusion domain of the wt G-protein and a targeting domain that contains a single-chain antibody specific for the Her2/neu receptor (SCA Her2).

Initially we tested mutant G-proteins indirectly by placing them on the surface of a pseudo-Moloney murine leukemia virus that expressed the green fluorescent protein (GFP) in infected cells. We then constructed a recombinant VSV (rVSV) in which the gene encoding the G protein was removed and a gene expressing GFP was added. G mutants were then tested directly in VSV pseudotypes. Mutations were created in the three positively charged amino acids found from amino acid positions 80 to 97 in the VSV-G protein. The mutant 92 GLU to ASP functioned normally. The mutants 87 ARG to PRO, 96 HIS to ASP, and 96 HIS to LEU had no function. The mutant 96 HIS to TYR had very reduced function but the defect in this mutant could apparently be complemented when a binding-only function was provided in trans by the hemagluttinin (HN) derived from SV-5 virus. These results suggest that the VSV-G mutant 96 HIS to TYR has reduction of binding capacity but retention of fusion capacity and thus can be complemented by the SV5-HN protein. In a separate approach utilizing the SV5 virus surface glycoproteins which already separate the binding and fusion functions, we demonstrated that we could express the SV5 F fusion protein and the SV5 HN binding protein on the surface of pseudo-VSV and achieve productive infection.

We have created chimeras which link SCA Her2 with either the entire VSV-G molecule, a linker and the TM portion of the VSV-G molecule, CD4, CD8, the TM region of an immunoglobulin heavy chain molecule and the TM region of the platelet derived growth factor receptor (PDGFR). Flow cytometric analysis has indicated that only SCA attached either to the entire VSV-G protein or the TM region of the VSV-G protein trafficked to the cell surface. Thus far, attempts to create viruses that combine the SCA binding function with either the SV5 F or the 96 HIS to TYR G mutant fusion function have not achieved selective infection of Her2/neu amplified cancer cells.

The present project wishes to develop a replication competent VSV which by selectively killing breast cancer cells and provoking an autoimmune inflammatory response to breast tissue eradicates both primary and metastatic breast cancer.

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CD34+ ENDOTHELIAL PROGENITORS AS CELLULAR VEHICLES FOR GENE THERAPY OF BREAST CANCER

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Angiogenesis, the process of forming new blood vessels from pre-existing vessels, promotes growth and metastasis of solid tumors. Furthermore, increasing intratumoral blood vessel density frequently correlates with tumor stage progression. Since the formation of new blood vessels is required to tumor development, angiogenesis offers a target for cancer therapy. Utilizing cells that are normally involved in the angiogenic process in vivo provides a means of targeting tumors. These "cellular vehicles" can be loaded with therapeutic agents that are subsequently delivered directly to the tumor. In this regard, a subset of circulating CD34+ hematopoietic cells, called endothelial progenitor cells (EPC), has been shown to migrate and incorporate into the endothelium of newly forming blood vessels. Compared to viral vectors, CD34+ EPCs have (i) low levels of immunogenicity and (ii) innate tropism for areas of angiogenesis, making them ideal cellular vehicles for targeting both local and disseminated neoplasms. We therefore developed a system utilizing CD34+ EPCs for delivering oncolytic adenovirus (Ad) vectors to tumor cells. First, the transduction efficiency of Ad type 5 was compared to that of two tropism-modified Ad vectors using CD34+ EPCs that were isolated from peripheral blood, which showed that a chimeric Ad vector with type 3 tropism was most efficient. Next, since Ad replication has not been demonstrated in CD34+ EPCs, we established that this cell type supports Ad replication and de novo virus production. CD34+ EPCs were then infected with oncolytic Ad vectors and co-cultured with a variety of tumor cells at different tumor cell:CD34+ EPC ratios. Depending on the target tumor cell type, as few as 1 infected CD34+ EPC per 10(5) tumor cells was sufficient to produce complete oncolysis of the tumor cell population. Using a cell migration model, we next confirmed that Ad infection does not perturb the chemotactic activity of CD34+ EPCs. The infected CD34+ EPCs were capable of migration toward an angiogenic environment and delivery of their oncolytic "payloads" to tumor cells. Taken together, these data demonstrate a novel use of cellular vehicles as anti-tumor agents. The utility of vector-loaded cellular vehicles for in vivo applications is currently underway.

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TARGETING A NOVEL VECTOR FOR BREAST CANCER GENE THERAPY

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We are testing the hypothesis that a model protozoan parasite gene therapy vector can be genetically altered to safely, specifically and effectively target breast cancer cells. We have developed transgenic T. gondii that stably express the suicide genes for Herpes Simplex Virus Type 1 thymidine kinase (TK) or bacterial cytosine deaminase. Our data indicate that trangenic T. gondii expressing these genes sensitize SKBR3 cells, that overexpress HER2/neu, to killing by ganciclovir or 5-fluorocytosine, respectively. Assessment by the DEAD red cell assay and by Trypan blue exclusion assays showed that infection of approximately 10 to 20% of the cell population with TK or CD expressing T. gondii, followed by treatment of the cell culture with ganciclovir or 5-fluorocytosine, respectively, resulted in greater than 90% killing of the uninfected cell population after a 24 h incubation. As expected, we find that the bystander effect is greater in the parasites which express both suicide enzymes. Only 5 to 10% of the SKBR3 cells need to be infected in order to kill >90% of the cells after treatment with ganciclovir and 5-fluorocytosine in the in vitro assays. We report the construction of a avirulent strain of T. gondii that does not cause disease in eith immune competent or immunocompromised mice. The development of a "safe" strain of T. gondii is important for considering the potential of this novel approach for possible future therapies. The avirulent mutant invades host cells normally and will express proteins for several days; however, this mutant does not replicate in vitro or in vivo in the absence of pyrimidine supplementation. A strategy for more specifically targeting the parasite to breast cancer cells was examined. The initial approach was to construct a bi-specific antibody linking the surface of the parasite to overexpressed HER2/neu on breast tumor cell lines. Our data show that this attachment approach improves the targeting of the parasite to SKBR3 tumor cells. Further engineering of this novel vector is required to improve the targeting efficacy and to further improve and evaluate the safety of the novel vector. We conclude that the use of T. gondii for tumor targeting presents some potential advantages over virus delivery vectors.

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BICISTRONIC CONDITIONALLY REPLICATION-COMPETENT ADENOVIRAL VECTORS WHICH ARE SELECTIVELY DIRECTLY LYTIC FOR BREAST AND OVARIAN CANCER CELLS

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Our laboratory has previously reported that the L-plastin transcriptional promoter can confer conditional replication competency of adenoviral vectors by placing the viral E1A gene (AdLpE1A), which is needed for viral replication, under the control of the L-plastin promoter. These vectors were shown to be directly cytolytic for breast and ovarian cancer cells both in vitro and in animal models, but were non-toxic for explants of normal ovarian and mammary epithelial cells. X.Y. Peng from our laboratory has recently reported in Cancer Research 61: 4405-4413, 2001 the use of replication incompetent adenoviral vectors which carry the L-plastin transcriptional promoter driven cytosine deaminase transcription unit (AdLpCD) to sensitize breast and ovarian cancer cells to the effects of chemotherapy both in experiments with cell lines and in vivo in animal models. These vectors were not toxic to normal epithelial cells. Lixin Zhang of our laboratory has now created an adenoviral vector (AdLpCDIRESE1A) in which both of the cytosine deaminase and the E1A viral genes have been lined by a IRES element and placed under the control a L-plastin promoter an adenoviral vector. This AdLpCDIRESE1A bicistronic vector was shown to be more toxic for cancer cell lines exposed in vitro to chemotherapy than was the AdLpCD or AdLpE1A single gene vectors, or to cells exposed to the AdLpCDIRESE1A vector in the absence of chemotherapy. In addition, the effect of intratumoral injection of the AdLpCDIRESE1A vector on the growth of an established human cancer cell line in SCID mice given daily intraperitoneal injections of 5Fluorocytosine (5FC) was compared to the effect of the AdLpCD vector and the AdLpCDIRESE1A vector in animals not given 5FC These experiments showed that the bicistronic adenoviral vectors suppressed the growth of human cancer cell lines in a SCID mouse to a greater degree than did the single transcription unit vectors. These vectors are undergoing additional preclinical study to prepare the way for a clinical trial, including work with cell lines and animal models.

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ADENOVIRUS VECTOR TARGETING TO BREAST CANCER BY SOLUBLE ADAPTER PROTEINS

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The use of adenovirus (Ad) vectors for cancer gene therapy applications is currently limited by several factors including broad Ad tropism associated with the widespread expression of coxsackievirus and adenovirus receptor (CAR) in normal human tissues, as well as limited levels of CAR on malignant cells. To target Ad to relevant cell types we have proposed employment of soluble CAR (sCAR) ectodomain fused with a ligand to block CAR-dependent native viral tropism and simultaneously achieve infection through a novel receptor overexpressed on cancer cells. We showed that most of tested cancer cell lines established from mammary gland display low levels of CAR while moderately express av-integrins and rather high levels of c-erbB-2 oncoprotein. To target Ad vectors to breast cancer cell types expressing av-integrins we engineered adapter proteins containing sCAR fused with either RGD4C or NGR peptide motif which are proven to have high potential for bacteriophage targeting to human tumor xenografts in mice model. Specifically, we designed the genes encoding sCAR, purification tag, phage T4 fibritin polypeptide, and a hinge region followed by a ligand sequence. Incorporation of fibritin polypeptide provided trimerization of sCAR fusion proteins that resulted in augmented affinity to Ad fiber knob domain and increased ability to block CAR-dependent Ad infection compared to monomeric sCAR protein. The use of sCAR-RGD4C adapter protein to mediate Ad infection of breast cancer cells allowed several-fold increase of gene transfer efficiency compared to Ad alone or virus complexed with sCAR-fibritin control protein. To confer Ad targeting capability to cells expressing the c-erbB-2/HER-2/neu oncogene we engineered sCARfC6.5 protein adapter containing C6.5 scFv to c-erbB-2 oncoprotein. We have demonstrated that sCARfC6.5 protein binds to cellular c-erbB-2 oncoprotein and mediates efficient Ad targeting via a CAR-independent pathway. As illustrated on breast cancer cell lines overexpressing c-erbB-2, Ad complexed with sCARfC6.5 targeting adapter provided up to a 130-fold enhancement of gene transfer efficiency. The use of trimeric sCAR-ligand protein adapters may augment Ad vector potency for targeted delivery of therapeutic genes into breast cancer cell types.

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INCORPORATION OF THE EGF-LIKE DOMAIN OF HEREGULIN INTO THE E2 GLYCOPROTEIN MODIFIES THE TROPISM OF SINDBIS VIRUS

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The future progress of cancer gene therapy relies on the development of efficient and safe vectors that can deliver therapeutic genes specifically to tumor cells. Using a replication-competent viral vector targeted to tumor cells may be the most efficient way of specifically killing a large number of malignant cells. We intend to develop Sindbis virus (SV), an alphavirus which contains a single-stranded positive sense RNA genome, into a targeted replication-competent viral vector for breast cancer gene therapy. Since SV kills cells by apoptosis, specific destruction of tumor cells will occur if the virus is targeted to breast cancer cells.

Alphaviruses have been developed as vectors for gene expression and are being developed as possible vaccine and gene delivery vectors for human infections and diseases. Alphaviruses are able to infect a broad range of vertebrate and invertebrate cells. To safely and efficiently prevent infections and treat diseases, a target cell-specific alphavirus vector is highly desirable.

To target SV, the prototype alphavirus, to receptors expressed on breast cancer cells, a putative receptor-binding domain of the SV E2 transmembrane glycoprotein was replaced with the epidermal growth factor (EGF)-like domain of the EGF-like factor, heregulin. Heregulin has affinity for the human EGF receptors, erbB-3 and erbB-4, which are expressed on certain breast cancer cell lines. After transfection of the human breast cancer cell line, SK-BR3, and the baby hamster cell line, BHK-21, with the heregulin-containing SV RNA, significant cytopathic effect and cell killing occurred only in the transfected SK-BR3 cells. The presence of the heregulin EGF-like domain in the E2 glycoprotein enabled SV to kill breast cancer cells but not BHK-21 cells. Alphaviruses that selectively kill tumor cells will enhance the potential of using alphavirus vectors to treat and prevent human diseases.

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QUASI-EQUIVALENT IMAGING AND TARGETING ASSEMBLIES DERIVED FROM POLYOMAVIRUS VIRION PROTEINS: POTENTIAL IN IMAGING AND GENE THERAPY

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We are developing novel assemblies that utilize the principle of "quasi-equivalence", i.e., the self assembly of containers, such as virus capsids, by large numbers of identical protein subunits. Because of their high polyvalency, such assemblies can achieve very high specificity from subunits that bind targets with low affinities. This principle is demonstrated by murine polyomavirus, that normally binds to glycoproteins containing sialic acids and to integrins on the surface of cells. The affinity of polyomavirus for sialic acid is low (kd ~ 1-5 mM), but when multiple receptors are engaged, a strong cooperative interaction occurs (kd ~ 20 pM). These properties can be employed to develop high-affinity imaging and targeting assemblies with novel specificities. By combining different protein subunits with affinities for several targets into one assembly, it should be possible to target with very high specificity, rare neoplastic cells among the large repertoir of normal cells present in an organism.

The relative ease with which polyomavirus VP1 quasi-equivalent assemblies (VLPs) can be expressed and purified has prompted us to develop them as vectors for gene therapy and as polyvalent carriers of imaging and therapeutic radionuclides. We have introduced sequences into the surface-exposed loops of VP1 that will target VLPs to receptors frequently expressed on breast cancer cells. We have introduced into VP1 loops BC, DE, EF and HI, sequences capable of binding to the urokinase plasminogen activator (uPA) receptor or the ErbB2 receptor. Introduction of these sequences renders most of the modified VP1 proteins insoluble when expressed at high levels in insect cells, although some modified proteins such as VP1/EF-uPA(10-34), -uPA(1-60) and -ErbB26.1 are partly soluble. Conditions have been developed to improve yield and solubility. The efficiency of self-assembly of these modified VP1 proteins appears to be reduced as compared to VP1/wt protein, however co-expression with VP1/HI-FLAG improves yields. That the VLPs contain the co-expressed VP1 proteins has been established by demonstrating VP1/EF-uPA(1-60) proteins migrating at the position of native VLPs in sucrose velocity sedimentation and co-precipitation with VP1/EF-FLAG proteins captured by anti-FLAG antibody. Specificity for receptors present on cancer cell surfaces has been defined. The VP1/EF-Flag proteins have been conjugated with an N-teminal chelate to which IN-111 was added, and the biodistribution and clearance in mice determined. Most VP1 assemblies are cleared rapidly from tissues, except for the liver.

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P53 EXPRESSION FROM A REPLICATION-COMPETENT ADENOVIRUS IMPROVES BREAST TUMOR CELL KILLING—DELETION OF THE DEATH PROTEIN IMPROVES SPECIFICITY

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Tumor-targeted replication-competent adenoviruses are being investigated for various cancers, but their intrinsic oncolytic activity is insufficient for effective therapy. Gene transfer of p53 induces cancer cell death, and replication-defective adenoviral vectors expressing p53 are being evaluated in clinical studies. However, low transduction efficiency limits the efficacy of replication-defective vectors. To improve efficacy and specificity, we have constructed a replication-competent adenovirus (Adp53rc) that expresses p53 late in the viral life cycle, and also contains a deletion of theE3-encoded death protein. Adp53rc infected p53-negative cancer cells expressed high levels of p53 in parallel with the late expression pattern of the adenoviral fiber protein. p53 expression did not impair effective virus propagation. Replication of Adp53rc, measured by plaque assay, was similar to replication of an identical p53-negative control virus (Ad-co) or wild-type virus (Ad309). Survival of two p53-negative breast cancer cell lines (HCC1428, HCC202), evaluated by WST-1 assay, was diminished after infection with Adp53rc compared to Ad-co. Interestingly, p53 expression in a death-protein-deleted viral backbone confers some tumor-specific cell killing. Adp53rc killed cancer cells more effectively than did the death-protein-expressing Ad309. In contrast, normal fibroblasts (IMR-90) were significantly less sensitive to Adp53rc than to Ad309 infection. In conclusion, p53 expression late in the life cycle of a replication-competent adenovirus improves tumor cell killing. In a death-protein-deleted viral backbone p53 expression confers some specificity of tumor cell killing. The development of replicating adenoviruses that can both effectively and specifically kill cancer cells, and spread through a tumor may lead to an effective therapy for breast cancer.

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PRO-APOPTOTIC AND ANTITUMOR ACTIVITIES OF ADENOVIRUS-MEDIATED p202 GENE TRANSFER

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We have being studying the pro-apoptotic and anti-tumor activity of p202, an interferon-inducible protein and its therapeutic efficacy for gene therapy in breast cancer animal model. To study the immediate effect of p202 and to test the potential efficacy of p202 treatment, we have established adenovirus-p202 (Ad-p202) and CMV-p202/SN2 (liposome) delivery systems to test its cell killing activity *in vitro*. We have found that Ad-p202 infected breast cancer cells have exhibited growth inhibition and sensitized to TNF-α, Taxol, CDDP or γ-irradiation induced apoptosis. In addition, we have demonstrated that for the first time Ad-p202 infection induces apoptosis, and that requires the activation of caspase-3 for full apoptotic effect. More importantly, we have shown the efficacy of Ad-p202 or CMV-p202/SN2 treatment on breast cancer xenograft model, and this anti-tumor effect correlated well with enhanced apoptosis in Ad-p202 or CMV-p202/SN2-treated tumors. Taken together, we have concluded that Ad-p202 (or CMV-p202/SN2) is a potent growth inhibitory, pro-apoptotic and tumor-suppressing agent. Ad-p202 (or CMV-p202/SN2) may be further developed into an efficient therapeutic agent for human breast cancer gene therapy.

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TUMOR REGRESSION BY PROHIBITIN RNA IN A METASTATIC RAT MAMMARY TUMOR MODEL

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The 3'untranslated region (3'UTR) of the prohibitin gene codes for an RNA that inhibits the G1 to S transition in the cell cycle. Some breast cancer cell lines have mutations in the prohibitin 3'UTR and these mutated RNAs have lost the ability to inhibit cell proliferation. In such cell lines, proliferation can be arrested by the introduction of wild type prohibitin 3'UTR. These results suggested that the prohibitin regulatory RNA is a tumor suppressor and might be of therapeutic value for breast cancer.

We tested this hypothesis by directly injecting prohibitin 3'UTR RNA into palpable tumors of a transplantable, metastatic mammary tumor model grown on Wistar-Furth rats. By day 12 after initial treatment, tumors receiving prohibitin RNA delivered in isotonic saline were significantly reduced in size compared to tumors receiving only isotonic saline or a control RNA. Approximately 50% of prohibitin RNA treated animals exhibited complete regression of tumor and disease-free, long-term survival when isotonic saline was used as carrier. Delivery of prohibitin RNA complexed with lipid (DOTAP) improved long-term survival. Prohibitin 3'UTR RNA/DOTAP complexes caused complete regression in 75% of treated animals. A total of 50 tumor-free, long term survivors were rechallenged with a new inoculate of tumor cells and 48 remained disease free. cDNA array analyses of alterations in gene expression that occur during tumor regression in this animal model were used to determine the mRNA expression profiles from regressing, treated tumors compared to control tumors. We identified decreased expression of several cell cycle related genes and increased expression of genes involved in apoptosis in the regressing tumors. Other genes not previously implicated to have a role in cancer also exhibited significant alterations in transcript levels.

We have shown that prohibitin 3'UTR RNA is able to effectively control tumor cellular proliferation in vivo when administered as a therapeutic and that it induces a systemic anti-tumor immunity in this rat model. The application of cDNA array analyses to molecularly dissect the pathways activated and repressed during tumor regression may aid in the identification and development of new therapeutic interventions for breast cancer.

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A NOVEL SYSTEM FOR TARGETING GENE DELIVERY TO BREAST CANCER CELLS

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HER2-positive breast cancers are highly aggressive, frequently resistant to chemotherapy, and associated with a high incidence of mortality. Therefore, therapy by alternative means, such as targeted delivery of therapeutic genes, may prove much more effective on these types of cancers than standard methods of treatment. Our work has focused on the development and testing of a novel gene delivery system that uses the highly evolved cell-entry functions of the fiber and penton proteins, which are derived from the Adenovirus serotype 5 (Ad5) capsid. The fiber protein, which is responsible for high affinity cell binding and contributes to intracellular trafficking, has been engineered to target breast cancer cells by the replacement of the wild-type receptor binding domain with that of the breast cancer cell specific ligand, heregulin. The penton protein, which is responsible for cell entry and endosomal lysis, has been engineered to transport DNA by appendage of a polylysine sequence. The assembly of these two proteins into non-viral targeted gene delivery complexes and the characterization of such complexes has been the focus of our research.

Our findings show that non-viral gene delivery complexes may be formed using recombinant engineered Ad5 capsid penton proteins. These complexes mediated gene transfer in culture by integrin receptor binding and internalization, and, with the appendage of a heregulin targeting ligand, could target gene delivery specifically to heregulin receptors on breast cancer cells. Heregulin receptor binding and endocytosis specifically mediated this delivery into cells and the penton protein is required for enhanced delivery.

This system would require only the minimal adenoviral proteins necessary for cell surface binding and internalization, and lacks all other viral proteins and genes. Thus, this system avoids the concerns associated with using viruses such as mutation and viral recombination. The targeted nature of this system avoids hair and blood cells, thus providing a safer alternative to standard methods of treatment.

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REOVIRUS AS AN ANTI-BREAST-CANCER AGENT

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We have previously shown that human reovirus replication is restricted to cells with an activated Ras pathway, and could be used as an effective oncolytic agent against human glioblastoma xenografts. This study examines in more detail the feasibility of reovirus as a breast cancer therapeutic, a subset of cancer in which direct activating mutations in the *ras* proto-oncogene are rare, yet where unregulated stimulation of Ras signaling pathways is important in the disease's pathogenesis. We show here that breast tumor-derived cell lines are permissive to the virus, whereas normal breast cells resist infection *in vitro*. *In vivo* studies of reovirus breast cancer therapy reveal that viral administration could cause tumor regression in an MDA-MB-435S mammary fat pad model in SCID mice. Reovirus could also effect regression of tumors remote from the injection site in an MDA-MB-468 bilateral tumor model, raising the possibility of systemic therapy of breast cancer by the oncolytic agent. Finally, the ability of reovirus to act against primary breast tumor samples not propagated as cell lines was evaluated; we found that reovirus could indeed replicate in *ex vivo* surgical specimens. Overall, reovirus shows promise as a potential breast cancer therapeutic.

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CONTROLLABLE GENE THERAPY FOR HUMAN BREAST CANCER

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One of the most vexing challenges facing cancer therapy is the difficulty of bringing therapy to poorly profused tumor areas without flooding the patient with lethally high doses of therapeutic agents. We propose to develop and evaluate an innovative approach using a combination of stress-inducible suicide gene therapy and hypoxia-inducing agents. Our plan is to turn the inaccessibility of poorly profused tumor areas into a selective mechanism for targeting these areas. Cancer gene therapy offers an attractive alternative towards the cure of cancer. It is relatively non-toxic in comparison to conventional systemic chemotherapy. However, a stumbling block in the development of anti-cancer therapy is the lack of specificity in targeting expression of suicide genes at the tumor sites. This proposal addresses this limitation by employing a novel approach to use a stress-inducible promoter from the grp78 gene to direct the expression of the suicide gene. The glucose-regulated protein gene (grp78) encodes for a molecular chaperone GRP78, also referred to as BiP, with anti-apoptotic properties. We and others have discovered that this class of glucose-regulated genes is turned on when human cells are deprived of glucose and oxygen, a hallmark of fast growing solid tumors with the lack of sufficient blood supply. Our strategy is to target suicide gene expression for treatment of breast tumors by taking advantage of their own physiological abnormalities, such as hypoxia, acidic pH and depletion of glucose. Our experimental approach includes developing an optimal gene control cassette that contains the most potent control elements for expression in a tumor environment with minimal expression in non-malignant cells. We further propose to combine suicide gene therapy driven by the grp78 promoter with photodynamic therapy (PDT) which is currently used in clinical trials for breast cancer treatment.

Using the human breast adenocarcinoma cell line MDA-MB-435 as a model system, we show that the grp78 promoter exhibited the desirable characteristic of low basal level expression as compared to the viral LTR, and high inducibility following stress. Through the use of PET imaging, we provide evidence of spontaneous, *in vivo* activation of the grp78 promoter in growing breast tumors and its direct activation by PDT in a controlled manner. In the context of a retroviral vector, the rat grp78 promoter-driven Herpes Simplex Virus thymidine kinase is fully active in the MDA-MB-435 human breast cancer xenografts, leading to complete tumor regression following ganciclovir treatment. Transcriptional targeting through the use of grp promoters, in combination with transduction systems designed for tumor delivery, can be a useful tool for breast tumor eradication.

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CHEMOSENSITIZATION THROUGH ADENOVIRUS E1A-MEDIATED SIGNAL INTEGRATION

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Most chemotherapeutic drugs kill cancer cells by inducing apoptosis and a deficiency in apoptosis often confers drug resistance. Therefore, a novel strategy for combating cancer is to restore the sensitivity of cancer cells to apoptosis.

By studying of stable cells in vitro and systemic gene therapy in othortopic breast cancer model in animal in vivo, we showed that introducing adenovirus type 5 early region 1A (E1A) into tumor cells resulted in sensitization to paclitaxel (Taxol)-induced apoptosis and enhancement of the Taxol antitumor activity. Further molecular analysis indicated that this beneficial anti-tumor effect was achieved, at least partly, by E1A-mediated signal integration through downregulating Akt activity and upregulating p38 activity in breast cancer cells. The same mechanism was observed using additional anti-cancer drugs, such as doxorubicin (Adriamycin), cis-platin, methotrexate, and gemcitabine. Further genetic analysis reveiled that disrupting Rb pathway by E1A was required for chemosensitization, whereas disrupting E1A's ability to bind with p300 did not abrogate E1A-mediated chemosensization.

Thus, the current study not only identified a novel mechanism for sensitization to chemotherapeutic drug-induced apoptosis, but also provided feasibility for further development of gene therapy and chemotherapy to treat cancer patients.

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A NOVEL THERAPY FOR BREAST CANCER BY INDUCING HYPERACUTE REJECTION

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This project presents an innovative approach to breast cancer gene therapy that exploits a naturally occurring physiologic process in humans. We propose that the alpha (1,3) galactosyltransferase [a (1,3) GT] gene in fact represents an ideal unconventional "suicide" gene to induce destruction of the tumor, because the mediators of cell death are inherent in the human immune system and not dependent on pharmacological intervention. Strong immunological barriers to xenotransplants from lower mammals into humans can destroy a transplanted solid organ within minutes, a process termed hyperacute rejection. The expression of the murine a(1,3)GT gene results in the cell surface expression of a(1,3)galactosyl epitopes (agal) on membrane glycoproteins. These epitopes are the major target of the human hyperacute rejection response that occurs when organs are transplanted from nonprimate donor species. a(1,3)GT is expressed in all mammals including Mus musculus, but not in old world primates, apes or humans. We employed a novel Herpes amplicon vector (HE7agal1) that efficiently infects human solid tumor cells at low multiplicity of infection and permits high-level transgene expression. The anti-tumor effectiveness of a(1,3)GT gene transfer has now been show in vitro in human breast tumor cell lines with rapid killing after normal human serum exposure by complement lysis. The a(1,3)GT knockout mouse serves as the only small animal model analogous to humans in which to study the immune response to the agal epitopes. a(1,3)GT KO mice immunized with rabbit RBC (agal+) produced high-titer anti-agal Ab responses in all mice. When agal immunized mice were challenged by injection with agal positive tumor cells, 70% to 100% of the mice survived up to 30 days. Control normal mice (agal+) challenged with the same tumor died uniformly before day 19 after challenge. Therefore, the presence of anti-agal Ab was highly protective. Next, a(1,3)GT KO mice were administered carcinogens to generate a murine breast cancer cell line (agal negative) for an in vivo tumor model. A murine breast cancer cell line (agal negative) was derived from these mice. In conclusion, our data show effective use of the murine a(1,3) GT gene as a therapeutic transgene to induce hyperacute rejection of breast cancer.

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NOVEL APPROACHES TO DESIGN OF TRIPLE HELIX-FORMING OLIGONUCLEOTIDES TO INHIBIT EXPRESSION OF C-MYC IN BREAST CANCER CELLS

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Selective targeting of genes that cause growth of breast cancer is an attractive therapeutic strategy. One approach uses triple helix-forming oligonucleotides (TFOs) directed to regulatory sequences in gene promoters to disrupt transcription and reduce expression of targeted genes. TFOs can bind either parallel or antiparallel to the purine-rich strand of polypurine/polypyrimidine sequences in double-stranded DNA. Properties of the target sequence determine the preferred orientation. The aim of our studies was to design high-affinity TFOs to inhibit transcription of the c-myc oncogene in breast cancer cells. Previous results suggested that TFOs targeted to a critical regulatory region close to the major P2 promoter could inhibit c-myc expression in cells. However, these TFOs had moderate binding affinity and required high concentrations for activity. We found that two discrete regions of the target sequence were maximally bound by TFOs with opposite orientation. To improve activity, we examined alternative TFO designs. We synthesized a parallel/antiparallel TFO with a central 3'-3' interface. Footprinting and EMSA showed that the novel TFO formed triple helix with higher binding affinity than conventional TFOs. In gel shift assays with breast cancer cell nuclear extracts, the TFO blocked binding of transcriptional activators Sp1 and Sp3 to the c-myc P2 sequence. To further optimize TFO activity we investigated effects of conjugation with a DNA intercalating agent. Binding studies showed that TFOs conjugated to the anthracycline antibiotic daunomycin (Dnm) formed more stable triple helix than their unconjugated counterparts. In shift assays with nuclear extracts Dnm-TFOs inhibited binding of proteins to the target duplex more efficiently than unconjugated TFOs. Furthermore, Dnm-TFOs were efficiently internalized by MCF-7 cells, as determined by fluorescence microscopy, and at nanomolar concentrations, inhibited expression in breast cancer cells of a c-myc promoter-driven luciferase gene. These results provide strategies for design of improved c-myc-targeted TFOs. Advances in TFO design bring this selective therapeutic strategy closer to testing in vivo and to future application in breast cancer treatment.

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AN ENDOTHELIAL CELL-BASED GENE THERAPY APPROACH FOR TREATMENT OF BREAST CANCER METASTASES

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Metastasis is the primary factor responsible for mortality in Breast cancer patients, and as yet effective means of treatment are elusive. In this study, we evaluated a new therapeutic approach for the treatment of breast cancer metastases in which endothelial cells are transfected in vitro with therapeutic genes and then injected into animals with cancer metastases. These modified endothelial cells seed to blood vessels supplying the tumor metastases and secrete the antitumor agent. To test this approach, we prepared mouse endothelial cells that express human interleukin 2 (hIL-2/GMEC), a potent promoter of the immune response. Then, breast tumors were established in BALB/c mice by injecting 100,000 syngeneic 4T1 cells, a metastatic murine mammary tumor cell line, into their mammary fat pads. Starting ten days later, a time when tumor metastases became fully established in the lungs of the animals, the mice were given three intravenous (IV) injections of 100,000 hIL-2/GMEC, spaced 3 days apart. At weekly intervals thereafter, groups of experimental and control mice were sacrificed and their lungs examined for the number of tumor foci and cellular infiltrates.

The untreated group of tumor-bearing mice had an average of 200 metastatic foci in their lungs and a median survival time of 21 days. Neutrophils and macrophages were confined to the edges of the tumors. In contrast, the hIL-2/GMEC-treated group of tumor-bearing mice had an average of 50 tumor foci in their lungs. They exhibited prolonged survival with a median of 40 days. The tumors in these mice were positive for human interleukin-2 and extensive infiltration of granulocytes, macrophages, and both CD4- and CD8-positive lymphocytes at both the periphery and center of the tumors. Non-tumor-bearing mice that had been given three IV injections of 100,000 hIL-2/GMEC remained alive and well. These results suggest that: 1) administration of hIL-2/GMEC can inhibit the growth of established metastases of breast cancer and prolong the survival of the mice, and 2) systemic administration of genetically modified endothelial cells does not cause obvious adverse effects in mice. This approach may be particularly useful for targeting therapeutic genes to sites of breast cancer metastases throughout the body.

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A NOVEL RNA VIRUS SYSTEM FOR SELECTIVE KILLING OF BREAST CANCER CELLS

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The paramyxovirus simian virus 5 (SV5) is a noncytopathic RNA virus with inherent properties which could be exploited to generate novel viruses for tumor therapy. Our goals are to develop a novel method for targeting an SV5 infection to breast cancer cells and to control the virus-mediated killing. These goals were approached in two parts: 1) engineer a SV5 vector to express an anti-HER-2 single chain antibody in place of the normal HN attachment protein, and 2) to engineer a SV5 vector to express the herpes simplex thymidine kinase which converts the prodrug gancyclovir to a toxin that kills cells.

Plasmids were constructed to encode chimeric proteins composed of an anti-HER-2 single chain antibody (sFv) linked to segments of the SV5 HN protein. Expression of the chimeric proteins in cells and cell surface immunofluorescence showed that transport of the chimeric HN-sFv proteins to the cell surface is not efficient, with only a very few cells showing bright staining. The genes for candidate fusion proteins have been inserted into the SV5 infectious clone in place of the gene for HN and we are attempting to recover these novel viruses. To determine if SV5 could be used for controlled cell killing, a recombinant SV5 (rSV5) was generated such that the thymidine kinase gene was inserted as an extra gene in the viral genome. Cells infected with rSV5-TK showed a time-dependent loss of viability when infected cells were cultured in the presence of the prodrug ganciclovir (GCV) while no significant toxicity was observed in the absence of prodrug. Titration experiments showed that rSV5-TK plus GCV resulted in cell death for all mouse and human cell lines tested, although the kinetics varied between cell types.

In summary, we have found that many of the HN-sFv fusion proteins are inefficiently transported to the cell surface. Future work will be focused on identifying approaches to improve the cell surface transport of HN-sFv molecules. Second, our results with rSV5-TK provide proof-of-concept that an inherently noncytopathic negative strand RNA virus can been engineered for killing cells in a controlled manner. Our results demonstrating controlled cell killing by a rSV5 support the further development of SV5 as a therapeutic vector for targeted killing of cancer cells.

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REPLICATION-COMPETENT HERPES SIMPLEX VIRUS VECTORS FOR THE TREATMENT OF BREAST CANCER

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We are developing a novel therapeutic strategy for the treatment of cancer that uses herpes simplex viruses (HSV) that are mutated so they replicate only in tumor cells and not in normal cells. These oncolytic replication-competent HSV vectors are cytotoxic to tumor cells, generating multiple new infectious progeny that are then able to infect and kill new tumor cells and spread in situ, yet are not pathogenic. In these studies, we examined the efficacy of a number of clinically-applicable oncolytic HSV vectors for the treatment of breast cancer in the C3(1)T-Ag transgenic mouse model. Breast cancer cell lines (M6, M6C) have been established from these mice and form tumors when implanted into transgenic mice.

There are currently two oncolytic HSV vectors in clinical trial in the US, G207 for recurrent glioma and NV1020 for colorectal cancer metastatic to the liver. G207 is a second-generation multi-mutated HSV vector, with deletions of both gamma34.5 loci, the major determinant of neuropathogenicity, and a LacZ insertion that inactivates the ICP6 gene, required for replication in non-dividing cells. NV1020 is an HSV-1/HSV-2 intertypic recombinant that contains a deletion of one copy of gamma34.5 and UL56. We have also tested G47Δ, derived from G207 with a deletion of ICP47 (inhibitor of MHC class I expression) that also results in early expression of US11 for enhanced viral replication, and NV1023 and NV1042, derived from NV1020 and expressing lacZ or IL12 respectively.

In vitro, G47Δ was the only vector effective at killing M6 cells and slightly less effective than NV1023 at killing M6C cells, with less than 40% of the cells surviving 3 days after infection at a multiplicity of infection (MOI) of 0.1. At this MOI, G207 was minimally cytotoxic. Neither G207 nor NV1023 were effective at inhibiting the growth of subcutaneous M6C tumors in C3(1)T-Ag heterozygous mice. G47Δ was more efficacious, similar to NV1042. A single injection of G47Δ into established intracerebral M6C tumors was able to extend the survival of these mice. Based on these results, we are planning to test G47Δ in the spontaneous breast tumors that develop in transgenic mice.

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A NOVEL SPATIALLY AND TEMPORALLY INDUCIBLE GENE EXPRESSION VECTOR FOR THE TREATMENT OF METASTATIC BREAST CANCER

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Systemic administration of combinations of cytotoxic agents is a standard therapy for patients with hormone-refractory breast cancer. High-dose chemotherapeutic regimens in general result in higher complete response rates compared to conventional dose regimens. However, a major obstacle to achieving higher complete response rates is the limitation of the dose of cytotoxic agents that can be administered because of systemic toxicity. A solution to this dilemma may be to find ways to confine the administration of cytotoxic agents to the tumors or their vicinity so that tumor cells are exposed to a higher level of the cytotoxic agents relative to other tissues. The goal of this proposal is to develop a novel gene expression vector to confine and enhance the expression of cytotoxic gene products to tumors cells in a spatial and temporal dependent manner for the treatment of breast cancer. To achieve this, a new vector, pBF (breast tumor specific, feed forward) was constructed to selectively enhance the level of cytotoxic agents at breast tumor sites relative to the rest of the body by using a feed-forward reaction based on a combination of tissue specific promoter (cerbB2p) and the tetracycline promoter (tetp) system. By placing the coding sequence for the cytotoxic agents and that of the fusion transactivator, TETON, under the control of both cerbB2p and tetp, a feedforward reaction is triggered in the presence of tetracycline in breast tumor cells in which cerbB2 is active but not in other cell types. The overall goal is to test and optimize the system to induce the expression of cytotoxic gene products in breast tumor cells at a high and sustained level with minimal background.

To test our hypothesis, we placed one, two and four copies of the cytotoxic agent, TNFα behind the tetp promoter. Transfection of these plasmids into cells showed that production of TNFα with two and four copies of the gene increased 1.4 and 3.8 fold over the one copy plasmid respectively. To further enhance the level of expression of TNFα, we placed tetp behind cerb2 so that the initial amount of tetON produced specifically in cerbB2 expressing cells will be further amplified. Comparison of this plasmid with one in which tetON is placed directly behind the cerbB2 promoter shows a 25 fold amplification.

In conclusion, we have shown that a high level of cytotoxic agent can be achieved by placing multiple copies of the agent behind the tetp promoter. Weakness of the breast tumor specific promoter cerbB2 can be overcome by placing a tetp promoter behind the cerbB2 promoter. Work is now in progress to transplant cells expressing these plasmids into SCID mice to study the level of background and steady state expression of TNFα in vivo. The validation of this gene expression vector will solve one of the major obstacles in the treatment of breast cancer (systemic toxicity) and should have significant impact on disease outcome.

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SPECIFIC EXPRESSION OF BCL-2 IN NORMAL CELLS TO PROTECT FROM RADIATION- OR CHEMOTHERAPY-INDUCED TOXICITIES

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Breast cancer patients receive a clear survival benefit from adjuvant radiation and or chemotherapy. In normal tissues, radiation or chemotherapy causes skin desquamation, mucositis, esophagitis, pulmonary fibrosis, cardiomyopathy, peripheral neuropathy which in turn can limit a patient's quality of life. The radiation or chemotherapy side effects on normal tissues are generally due to apoptosis in normal cells by their own inflammatory and immune responses to radiation or chemotherapy. Thus, we hypothesize that ectopic overexpression of anti-apoptotic molecules (Bcl-2) will inhibit the chemotherapy- and radiation-induced apoptosis of normal cells and thus reduce toxicities. We overexpressed Bcl-2 in murine fibroblast NIH3T3 and breast epithelial cells MCF10A which resulted in resistance to chemotherapy (Adriamycin or Taxol). We further prepared an heterogeneous plasmid that express luciferase cDNA in front of a minimal promoter regulated by multiple wild-type (wt) p53 DNA binding sites. Because the response of normal cells to genotoxic damage such as ionizing radiation can upregulate expression of wt p53 in such cells but not in cancers cells in which p53 is commonly mutated or deleted. When MCF10A or NIH3T3 is exposed to chemotherapy, p53 upregulation in these cells resulted in specific expression of luciferase. This may allow to protect normal cells expressing wt p53, but not p53-mutated or -deleted breast cancer cells, from radiation- or chemotherapy- induced side effects by inducing the wt p53-specific expression of Bcl-2.

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TRANSCRIPTIONAL TARGETING OF CONDITIONALLY REPLICATING ADENOVIRUS TO DIVIDING ENDOTHELIAL CELLS

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Conditionally replicating adenoviruses (CRADs) are a novel strategy in cancer treatment and clinical trials using CRADs targeted to tumor cells have been reported recently. We hypothesized that it would be possible to construct CRADs targeted to dividing endothelial cells, which are present in the tumor endothelium. We utilized the regulatory elements of Flk-1 and endoglin genes, which have been shown to be highly overexpressed in angiogenic endothelial cells, to construct two CRADs: Ad.Flk-1, which has adenoviral E1A gene under the control of the Flk-1 enhancer/promoter, and Ad.Flk-Endo, which harbors the same Flk-1 enhancer/promoter as Ad.Flk-1, plus it has the adenoviral E1B gene under control of the endoglin promoter. Viral titer measurements by plaque assay showed that in human umbilical vein endothelial cells (HUVECs), both CRADs replicated at levels comparable to that of wild-type adenovirus. In Flk-1 and endoglin negative Hep3B and A549 cells, however, the replication of Ad.Flk-1 and Ad.Flk-Endo was reduced by 30-fold and 600-fold, respectively. Cytotoxicity assays demonstrated that both CRADs killed HUVECs as effectively as wild-type adenovirus and their cytotoxicity in Hep3B and A549 cells was comparable to nonreplicating control adenovirus. Furthermore, there was a striking inhibition (83% - 91%) of capillary network formation in an in vitro angiogenesis assay when HUVECs were infected with Ad.Flk-1 or Ad.Flk-Endo as compared to the nonreplicating control virus. These results demonstrate that CRADs can be transcriptionally targeted to dividing endothelial cells with high specificity, and that the combined use of Flk-1 and endoglin regulatory elements has a synergistic effect on targeting specificity. This principle may be incorporated into novel therapeutic agents to develop anti-angiogenic treatment for cancer.

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TARGETING HER-2/NEU-OVEREXPRESSING BREAST CANCER CELLS BY AN ANTISENSE IRON-RESPONSIVE ELEMENT-DIRECTED BAX GENE EXPRESSION

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Overexpression of HER-2/neu proto-oncogene is found in many human cancers including 20-30% of breast cancer and is a predictor of poor prognosis. To target breast cancer cells that overexpress HER-2/neu mRNA, we previously described a novel strategy that combines the principle of antisense (AS) and translational inhibitory activity conferred by an iron-responsive element (IRE) (AS-IRE). Here, we showed that three potential AS-IREs, i.e., AS-IRE1, 4, and 5, derived from HER-2/neu antisense sequence could bind endogenous iron regulatory protein (IRP) and, when placed in 5' untranslated region (5'UTR) of a reporter gene, the gene expression could be translationally repressed by recombinant IRP in vitro. Using AS-IRE4 as our model, we demonstrated that it is regulated by iron, and importantly, such regulation is impaired in HER-2/neu-overexpressing breast cancer cells. Furthermore, we showed that AS-IRE4 could preferentially direct the expression of a reporter gene in HER-2/neu-overexpressing breast cancer cells. Interestingly, when AS-IRE4 was placed in 5'UTR of Bax gene, a pro-apoptotic protein in the Bcl-2 protein family, we observed a preferential cell killing in breast cancer cells that overexpress HER-2/neu. Taken together, our results suggest that AS-IRE behaves as a functional IRE and it may direct therapeutic gene expression to preferentially target HER-2/neu-overexpressing breast cancer cells.

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IMPROVING RETROVIRAL VECTORS FOR GENE THERAPY OF BREAST CANCER

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Conventional replication-defective murine leukemia virus (MLV)-based vectors have been unable to achieve effective transduction of solid tumors *in vivo*, and this has been a major obstacle in clinical trials of cancer gene therapy. Gene transfer using replication-competent retrovirus (RCR) vectors would be more efficient, as each successfully transduced tumor cell would itself become a virus-producing cell and thereby initiate further infection events. In contrast to various other replicating viruses now in development as cancer therapeutics, MLV-based RCR vectors can replicate without immediate lysis of host cells and can spread via direct cell-to-cell budding, and may be less likely to elicit robust immune responses that prematurely terminate virus propagation.

MLV-based RCR vectors for delivery of non-viral transgenes have been described previously; however, such vectors have usually exhibited rapid rearrangement and transgene deletion within one or two replication cycles. We have now devised a novel RCR vector design that has proven to be genetically stable over multiple serial replication cycles in cell culture, and can achieve highly efficient delivery of inserted transgenes to solid tumors *in vivo* in a variety of cancer models.

With intratumoral injection of RCR vectors at a multiplicity of infection (MOI) even as low as 0.001 to 0.01, over a period of several weeks it was possible to achieve almost complete transduction throughout an entire solid tumor. The RCR vector was undetectable in normal tissues by sensitive PCR assays, presumably due to selectivity for rapidly dividing tumor cells provided by the intrinsic inability of MLV to infect quiescent cells. To further enhance tumor selectivity and minimize the potential risk to normal cells, we have also tested two different strategies for targeting RCR vectors specifically to breast cancer cells, by 1) re-directing binding tropism through modification of the viral envelope to achieve HER2-specific adsorption to the cell surface, and 2) replacement of regulatory sequences in the LTR with tissue-specific or inducible elements in order to restrict viral transcription and replication to mammary tumors. Our results demonstrate that these strategies can be successfully employed to target RCR vectors to breast cancer cells, thereby minimizing any potential risk to normal cells while taking advantage of the powerful amplification process that results from viral replication, which results in a significant enhancement of transduction efficiency.

We have further designed and tested RCR vectors expressing a variety of suicide genes, which encode enzymes, such as yeast cytosine deaminase and *E. coli* purine nucleoside phosphorylase, that intracellularly convert non-toxic pro-drugs to toxic drug metabolites. We have confirmed that this strategy is successful in achieving highly efficient killing of breast cancer cells in culture as well as in mammary tumor models *in vivo*. After RCR vectors carrying the suicide genes have spread throughout the tumor, all infected cells are simultaneously killed by prodrug administration, resulting in significantly increased survival.

These results thus demonstrate the effectiveness of RCR vector-mediated suicide gene transfer, and underscore the potential of targeted RCR vectors as a safe and potent therapeutic modality for gene therapy of locally advanced or metastatic breast cancer.

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